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Published in:
Brain Research

DOI:
10.1016/0006-8993(91)91590-W

Brain Research, 542 (1991) 348–352
Elsevier

BRES 24553

Colocalization of muscarinic and nicotinic receptors in cholinoceptive neurons of the suprachiasmatic region in young and aged rats

E.A. van der Zee[1], C. Streefland[1], A.D. Strosberg[2], H. Schröder[3] and P.G.M. Luiten[1]

[1]*Department of Animal Physiology, University of Groningen, Haren (The Netherlands),* [2]*Laboratoire d'Immunopharmacologie Moléculaire, Institut Cochin de Génétique Moléculaire, Paris (France) and* [3]*Anatomical Institute, University of Cologne, Cologne (Germany)*

(Accepted 20 November 1990)

Key words: Aging; Circadian rhythm; Immunocytochemistry; Muscarinic receptor; Nicotinic receptor; Suprachiasmatic nucleus

In the present study muscarinic and nicotinic cholinergic receptors in the SCN region were demonstrated and analyzed, employing monoclonal antibodies to purified muscarinic and nicotinic cholinergic receptor proteins. A near-total colocalization of the two acetylcholine receptor subclasses in cholinoceptive neurons of the SCN area was found. The antibodies applied to aging rat brain (at 30–34 months) revealed a clear decrease in immunoreactivity in senescence albeit with a high level of individual variability. Furthermore, in 8 out of 10 aged animals examined a considerable increase of astrocytes possessing muscarinic cholinergic receptors was observed.

The suprachiasmatic nucleus (SCN) of the hypothalamus has been identified as a primary pacemaker for circadian rhythms in mammals and has been subject to extensive investigation[12]. Several studies point at a role of the neurotransmitter acetylcholine (ACh) in the time-keeping system of the SCN. It was shown that both the ACh-degrading enzyme acetylcholinesterase (AChE) and the ACh-synthesizing enzyme choline acetyltransferase (ChAT) are present in the SCN and its surrounding neuropil[1,6,10,17]. Furthermore, the cholinoceptive character of the SCN is indicated by the electrophysiological responsiveness of neurons to ACh in both in vivo and in vitro preparations[9,15]. Also, application of the non-selective cholinergic agonist carbachol shifts the circadian activity rhythms of rodents in a phase-dependent manner[3,13,25].

It is obvious that neurons in the SCN that receive a cholinergic innervation should possess cholinergic receptors of the muscarinic (mAChR) or nicotinic (nAChR) type, or both. Autoradiographic ligand binding studies utilizing tritiated quinuclidinylbenzilate (QNB)[8] or propylbenzilylcholine mustard (PrBCM)[19] and iodinated α-bungarotoxin (α-BTX)[4,16] revealed both muscarinic and nicotinic receptors in the SCN.

The present study was carried out to analyze the cholinoceptive characteristics of the SCN by immunocytochemical single and double labeling experiments employing monoclonal antibodies raised against purified mAChR-proteins and nAChR-proteins in the rat. As part of our general interest in the effect of aging on the cholinergic system[5] we also studied its receptors in the SCN in senescence. A preliminary report of this study was presented elsewhere[23].

The findings presented here were obtained from 10 young adult (3 months) and 10 aged (30–34 months) male Wistar rats. The animals had been housed individually or in small groups at a light/dark schedule of 12/12 h, with lights on at 8.00 h. Fixation of the brain was carried out in the early light period by transcardial perfusion with 300 ml fixative composed of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). Immediately upon fixation the fixative was removed by a perfusion with 150 ml 10% sucrose in 0.1 M PB. The brains were cryoprotected by overnight storage at 4 °C in 30% sucrose in 0.1 M PB and subsequently coronally sectioned on a cryostat microtome at a thickness of 20 μm.

Muscarinic and nicotinic receptor proteins were visualized by means of the monoclonal antibodies M35 and WF6, respectively. Extensive descriptions of production, characterization and immunocytochemical applications of these antibodies have been reported previously[20–22]. For single labeling, the brain sections were incubated 24–48 h at 4 °C in the primary antibody solution in phosphate-buffered saline (PBS) containing mouse IgM anti-mAChR (M35, 1:2000) or mouse IgG anti-nAChR

Correspondence: E.A. van der Zee, Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands.

(WF6, 1:10). After rinsing, the sections were exposed to biotinylated rabbit anti-mouse IgM (Zymed) or biotinylated sheep anti-mouse IgG (Amersham), respectively, followed by incubation in Streptavidin-HRP (Zymed). Double-labeling experiments for the study of colocalization of both receptor types were carried out with fluorescence techniques. For dual labeling, the sections were exposed to one of the primary antibodies as for single labeling. For WF6 the primary antibody step was followed by goat anti-mouse Phycoerythrin (Tago). After completion of the WF6 staining, the sections were incubated with M35 followed by biotinylated rabbit anti-mouse IgM and FITC-conjugated Streptavidin (Zymed). Standard control experiments for single labeling were performed by omission of the primary antibody step, or by replacing the primary antibody by normal mouse serum. In all cases the controls yielded negative results, i.e. absence of any detectable labeling.

Single labeling for muscarinic (M35) and nicotinic (WF6) acetylcholine receptors demonstrated the presence of both receptor types in cholinoceptive neurons in the SCN region (Fig. 1). In this area loosely scattered immunoreactive cells with their dendritic processes were found, however, without a clear rostrocaudal distribution pattern. The muscarinic and nicotinic cholinoceptive neurons were not restricted to the SCN boundaries, but were spread over the entire suprachiasmatic area. The number of immunoreactive cells was somewhat higher in the dorsolateral part of the region. Some immunolabeled cells were present within the optic chiasm. The highest density of cholinoceptive neurons was found in the middle portion of the SCN. In this region of the SCN, Cresyl violet-counterstained sections indicated that in single-labeling experiments approximately one out of 10 cells were immunoreactive for M35 or WF6. The cells showing immunoreactivity for M35 or WF6 predominantly had a somewhat elongated, bipolar morphology, although some round, multipolar cells have been found as well (Fig. 1).

Adjacent sections stained for M35 and WF6 revealed identical staining patterns and cell types, suggesting that the cholinoceptive cells in the SCN contain both types of acetylcholine receptors. Employing double-labeling fluorescent methods it became clear that nearly all cholinoceptive neurons in the SCN region possess both muscarinic and nicotinic receptors (Fig. 2). Only occasionally could single labeled neurons for M35 or WF6 be detected.

Immunoreactivity to muscarinic and nicotinic receptor proteins in single-labeling procedures was also investigated in 10 aged rats. In two aged animals we observed an almost complete lack of M35 and WF6 immunoreactivity in the SCN region, while in 3 other cases the

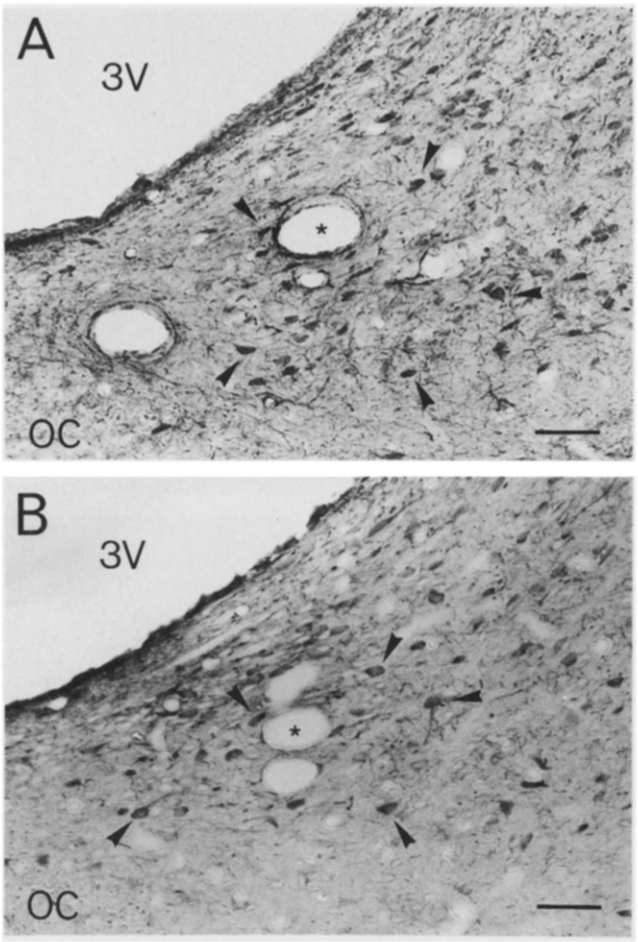

Fig. 1. Photomicrographs of the localization of cholinoceptive neurons in the SCN region. A: immunoreactivity to muscarinic receptor protein, showing cell bodies (arrowheads) and dendritic profiles. B: immunoreactivity to nicotinic receptor protein, approximately at an identical level of the SCN region, showing cell bodies (arrowheads) and dendritic profiles. Asterisks: blood vessels; OC, optic chiasm; 3V, third ventricle. Scale bar = 50 μm.

number and density of neuronal labeling was considerably decreased (Fig. 3). In 5 other animals, however, no obvious effect of aging on cholinergic receptor labeling could be detected with the currently used methods, indicating a high level of variation in aging. The observed decrease in immunoreactive cells in the SCN area in some of the senescent rats was not the result of technical failure such as reduced antibody penetration, since there was no difference in immunostaining in other cholinoceptive regions in the same sections. From these observations it may be concluded that the cholinergic receptors in the SCN area display a high susceptibility to the aging process, however, with a considerable individual variation.

Apart from the influence of aging on labeling with M35 and WF6 in neurons of the SCN, there was also a striking aging effect on M35 immunoreactivity in astrocytes. In

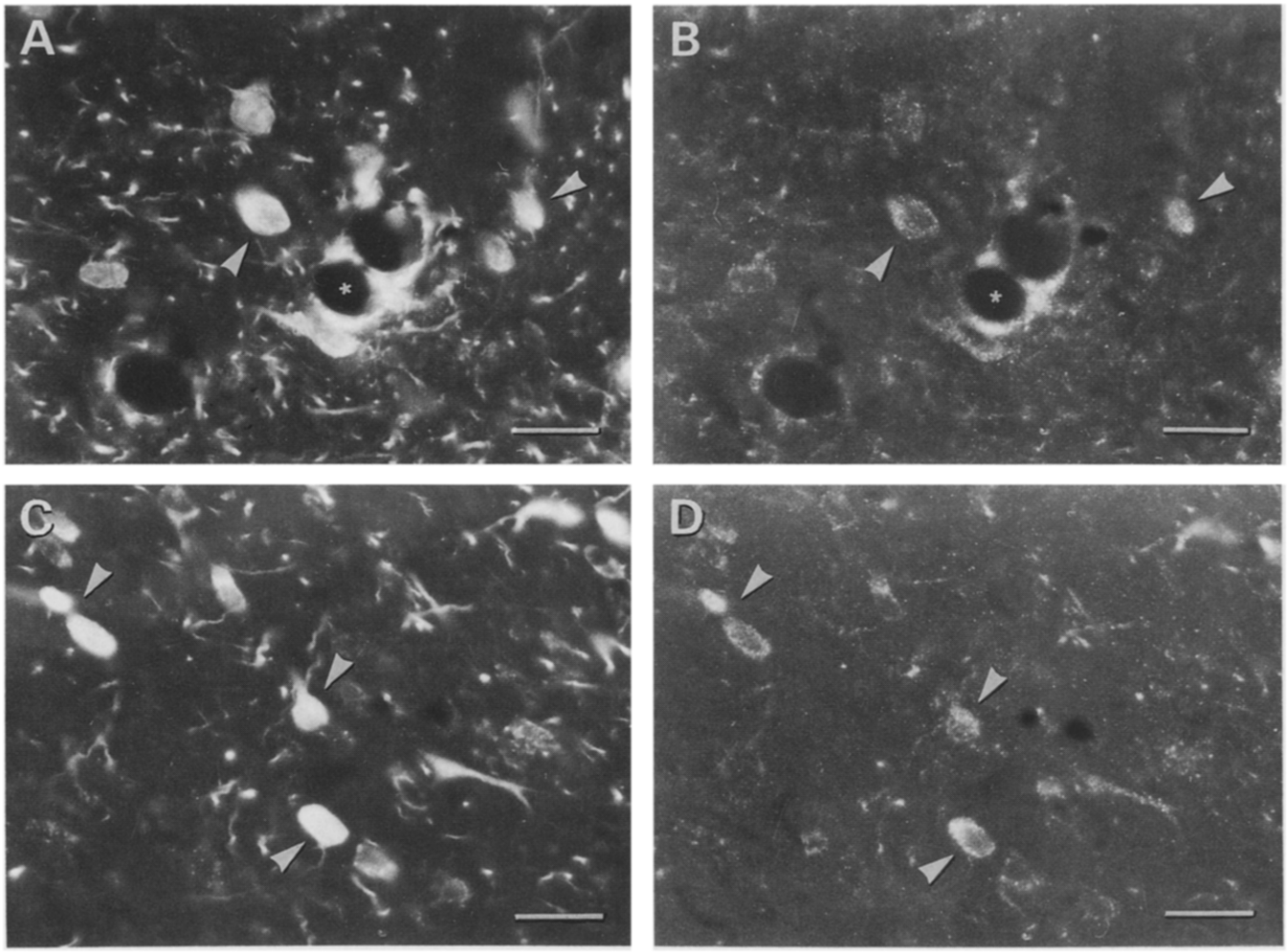

Fig. 2. Fluorescence images of double-labeling for muscarinic and nicotinic receptor protein in the middle portion of the SCN. Muscarinic cholinoceptive neurons are visualized by FITC (A,C), and nicotinic cholinoceptive neurons by Phycoerythrin (B,D). Neurons clearly displaying both receptor types are indicated by arrowheads. Asterisks: blood vessel. Scale bar = 25 μm.

young adult animals the staining of astrocytes with M35 is rare. In the aging brain, however, in 8 out of 10 animals examined, a considerable increase of M35-labeled astrocytes was observed (Fig. 3A).

The currently presented data obtained with immunocytochemical methods employing monoclonal antibodies to mAChR and nAChR proteins revealed both ACh receptor types to be present in neurons in the SCN area. These findings agree well with previous autoradiographic ligand binding studies of the SCN. Specific binding of muscarinic receptor ligands in the SCN and its surrounding neuropil was reported for tritiated QNB and PrBCM[8,19]. A similar pattern of high binding in the SCN and the adjacent dorsolateral SCN area is described for the nicotinic receptor antagonist α-bungarotoxin (α-BTX)[16]. Besides the results obtained with α-BTX, Mason[11] found stained processes and immunopositive neurons in the SCN using a monoclonal antibody, raised against the α-subunit of nicotinic receptors isolated from the electric eel. However, no detailed study was made of this nucleus.

Effects of cholinergic manipulation of the SCN acting through both types of ACh receptor subclasses have been reported. For example, the nicotinic antagonist mecamylamine blocks light-induced phase shifts in circadian activity rhythm in the golden hamster[7], and intravenous administration of nicotine has an excitatory effect on SCN cells[14]. Studies in brain slice preparations of the SCN reveal inhibitory effects on ACh application by the selective muscarinic antagonist atropine[9]. Since ACh as the endogenous messenger is a non-selective agonist for nicotinic and muscarinic receptors, both receptor types which are colocalized within the same neurons, will differentially contribute to the SCN timekeeping system.

In the brain of senescent rats, a striking effect of the

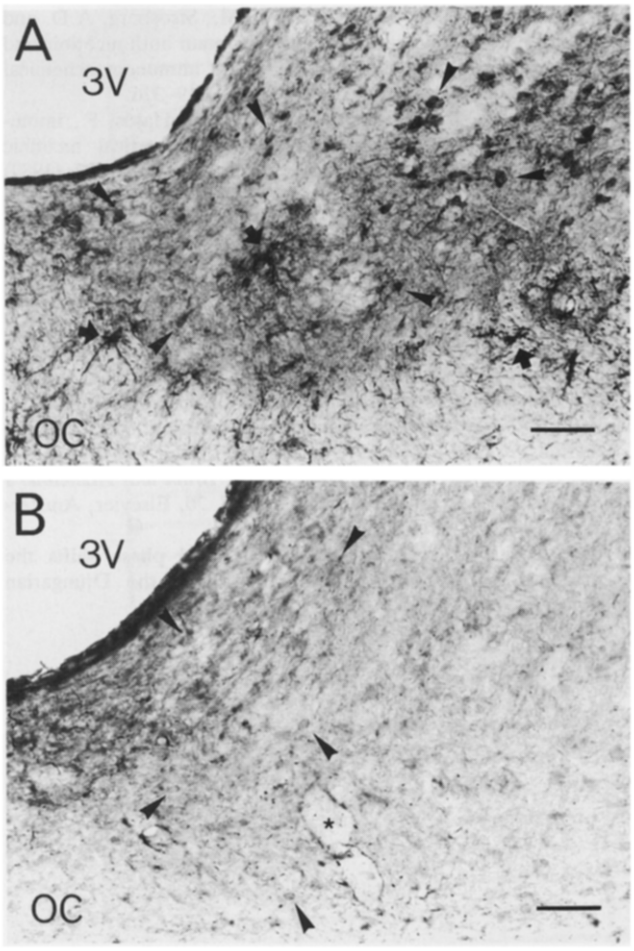

Fig. 3. Photomicrographs of muscarinic cholinoceptive neurons in aged rats. A: immunoreactivity for muscarinic receptor protein in neurons (arrowheads) of the SCN region decreased slightly. Increased numbers of immunoreactive astrocytes (arrows) were observed in most aged rats. B: in 3 of 10 aged rats only some weak-immunopositive neurons could be found (arrowheads). 3V, third ventricle; OC, optic chiasm; asterisk: blood vessel. Scale bar = 50 μm.

aging process on M35 and WF6 immunoreactivity in the SCN region could be observed in half of the animals studied. The effects, however, showed a high degree of individual variation ranging from a near total absence to only a minor deterioration of immunostaining. It remains unclear whether the decrease or lack of cholinergic receptor staining in part of the aging animals is the result of cell death or break-down of receptor protein. In agreement with earlier studies[2,18], there was no obvious decrease of total cell numbers in the SCN of our senescent rats, suggesting a specific aging-associated anomaly of cholinergic receptor expression. This tentative receptor break-down is clearly limited and thus characteristic of the SCN area, since it was not observed in other cholinoceptive regions in the same brain sections. The observed decrease in cholinergic receptor immunoreactivity in the SCN in part of the aged animals may be associated with the reported deterioration of the timekeeping system of the SCN leading to a deranged circadian rhythm[24].

In nearly all aged animals an increase of M35-immunoreactive astrocytes in the SCN was observed. Taking the number of immunoreactive astroglia cells into consideration it is evident, however, that only a minor proportion of astroglia, even in the aging condition, expresses immunoreactivity to muscarinic receptors. In young adult cases such astrocyte staining was mainly present in the superficial layers of cerebral cortex and hippocampus[22]. The function of cholinergic neurotransmitter receptors in astrocytes, however, remains unclear.

We wish to thank J.H. Meijer and B. Roozendaal for critically reading the manuscript. Part of this work was supported by a grant from Troponwerke (Bayer) to E.A.v.d.Z.

1 Brownstein, M.J., Kobayashi, R., Palkovits, M. and Saavreda, J.M., Choline acetyltransferase levels in diencephalic nuclei of the rat, *J. Neurochem.*, 24 (1975) 35–38.
2 Chee, C.A., Roozendaal, B., Swaab, D.F., Goudsmit, E. and Mirmiran, M., Vasoactive intestinal polypeptide neuron changes in the senile rat suprachiasmatic nucleus, *Neurobiol. Aging*, 9 (1988) 307–312.
3 Earnest, D.J. and Turek, F.W., Neurochemical basis for the photic control of circadian rhythms and seasonal reproductive cycles: role for acetylcholine, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 4277–4281.
4 Fuchs, J.L. and Hoppens, K.S., α-Bungarotoxin binding in relation to functional organization of the rat suprachiasmatic nucleus, *Brain Research*, 407 (1987) 9–16.
5 Gaykema, R.P.A., Gaál, G., Traber, J., Hersh, L.B. and Luiten, P.G.M., The basal forebrain cholinergic system: efferent and afferent connectivity, and long-term effects of lesions, *Acta Psychiatr. Scand.*, in press.
6 Ichikawa, T. and Hirata, Y., Organization of choline acetyltransferase-containing structures in the forebrain of the rat, *J. Neurosci.*, 6 (1986) 281–292.
7 Keefe, D.L., Earnest, D.J., Nelson, D., Takahashi, J.S. and Turek, F.W., A cholinergic antagonist, mecamylamine, blocks the phase-shifting effects of light on the circadian rhythm of locomotor activity in the golden hamster, *Brain Research*, 403 (1987) 308–312.
8 Kobayashi, R.M., Palkovits, M., Hruska, R.E., Rothschild, R. and Yamamura, H.I., Regional distribution of muscarinic cholinergic receptors in rat brain, *Brain Research*, 154 (1978) 13–23.
9 Kow, L.-M. and Pfaff, D.W., Suprachiasmatic neurons in tissue slices from ovariectomized rats: electrophysiological and neuropharmacological characterization and the effects of estrogen treatment, *Brain Research*, 297 (1984) 275–286.
10 Lauber, A.H., Miller, J.D., Murakami, D.M. and Fuller, C.A., Cholinoceptive neurons in the SCN, *Soc. Neurosci. Abstr.*, 10 (1984) 501.
11 Mason, W.T., Staining of the magnocellular nuclei of the rat hypothalamus by a monoclonal antibody directed against the α-subunit of the nicotinic cholinergic receptor, *Neurosci. Lett.*, 59 (1985) 89–95.
12 Meijer, J.H. and Rietveld, W.J., The neurophysiology of the suprachiasmatic circadian pacemaker in rodents, *Psych. Rev.*, 69 (1989) 671–707.
13 Meijer, J.H., Van der Zee, E.A. and Dietz, M., The effects of intraventricular carbachol injections on the free-running activity

rhythm of the hamster, *J. Biol. Rhythms*, 3 (1988) 333–348.
14 Miller, J.D., Murakami, D.M. and Fuller, C.A., The response of suprachiasmatic neurons of the rat hypothalamus to photic and nicotinic stimuli, *J. Neurosci.*, 7 (1987) 978–986.
15 Nishino, H. and Koizumi, K., Responses of neurons in the suprachiasmatic nuclei of the hypothalamus to putative transmitters, *Brain Research*, 120 (1977) 167–172.
16 Pauly, J.R. and Horseman, N.D., Autoradiographic localization of α-bungarotoxin binding sites in the suprachiasmatic region of rat brain, *Brain Research*, 452 (1988) 105–112.
17 Rao, Z.R., Yamano, M., Wanaka, A., Tatehata, T., Shiosaka, S. and Tohyama, M., Distribution of cholinergic neurons and fibers in the hypothalamus of the rat using choline acetyltransferase as a marker, *Neuroscience*, 20 (1987) 923–934.
18 Roozendaal, B., Van Gool, W.A., Swaab, D.F., Hoogendijk, J.E. and Mirmiran, M., Changes in vasopressin cells of the rat suprachiasmatic nucleus with aging, *Brain Research*, 409 (1987) 259–264.
19 Rotter, A., Birdsall, N.J.M., Burgen, A.S.V., Field, P.M., Hulme, E.C. and Raisman, G., Muscarinic receptors in the central nervous system of the rat. I. Technique for autoradiographic localization of the binding of the [^{3}H]propylbenzilylcholine mustard and its distribution in the forebrain, *Brain Res. Rev.*, 1 (1979) 141–165.
20 Schröder, H., Zilles, K., Luiten, P.G.M., Strosberg, A.D. and Aghchi, A., Human cortical neurons contain both nicotinic and muscarinic acetylcholine receptors: an immunocytochemical double-labeling study, *Synapse*, 4 (1990) 319–326.
21 Schröder, H., Zilles, K., Maelicke, A. and Hajós, F., Immunohisto- and cytochemical localization of cortical nicotinic cholinoceptors in rat and man, *Brain Research*, 502 (1989) 287–295.
22 Van der Zee, E.A., Matsuyama, T., Strosberg, A.D., Traber, J. and Luiten, P.G.M., Demonstration of muscarinic acetylcholine receptor-like immunoreactivity in the rat forebrain and upper brainstem, *Histochemistry*, 92 (1989) 475–485.
23 Van der Zee, E.A., Streefland, C., Luiten, P.G.M., Strosberg, A.D. and Zilles, K., Immunocytochemical demonstration of muscarinic and nicotinic acetylcholine receptors in the rat suprachiasmatic nucleus, *Annu. Meet. ENA*, 12 (1989) 120.
24 Van Gool, W.A. and Mirmiran, M., Aging and circadian rhythms. In D.F. Swaab, E. Fliers, M. Mirmiran, W.A. van Gool and F. van Haaren (Eds.), *Aging of the Brain and Alzheimer's Disease, Progress in Brain Research, Vol. 70*, Elsevier, Amsterdam, 1986, pp. 1811–1819.
25 Wee, B.E.F. and Turek, F.W., Carbachol phase shifts the circadian rhythm of locomotor activity in the Djungarian hamster, *Brain Research*, 505 (1989) 209–214.